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REVIEW

Single-cell and spatially resolved transcriptomics for liver biology

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Abstract

Single-cell transcriptomics enables the identification of rare cell types and the inference of state transitions, whereas spatially resolved transcriptomics allows the quantification of cells and genes in the context of tissues. The recent progress in these new technologies is improving our understanding of the cell landscape and its roles in diseases. Here, we review key biological insights into liver homeostasis, development, regeneration, chronic liver disease, and cancer obtained from single-cell and spatially resolved transcriptomics. We highlight recent progress in the liver cell atlas that characterizes the comprehensive cellular composition; diversity and function; the spatial architecture such as liver zonation, cell communication, and proximity; the cell identity conversion and cell-specific alterations that are associated with liver pathology; and new therapeutic targets. We further discuss outstanding challenges, advanced experimental technologies, and computational methods that help to address these challenges.

OVERVIEW

The liver is one of the most important metabolic organs in the body, which is highly organized, with the operation of hepatic functions heavily relying on its delicate architecture.[1] Liver disease is estimated to affect billions of people globally,[2,3] which is recognized in various forms, including chemical-induced liver injury, NAFLD, liver cirrhosis, and liver cancer. The pathogenesis of liver disease involves dramatic cell state transitions, extensive cell–cell interactions, and prominent histological changes that disturb the spatial architecture of the organ,[4] leading to the demand to decode the spatiotemporal programs of hepatic cells during the course of liver disease.[1,4–6]

Single-cell RNA sequencing (scRNA-seq) and spatially resolved transcriptomics are ideal approaches to understand liver biology in the following scenarios (Figure 1): (i) constructing the single-cell atlas of both homeostatic and diseased livers that comprehensively describes common, rare, and disease-associated subpopulations[5,7–9]; (ii) deciphering the molecular

Abbreviations: 10X Visium, 10X Genomics Visium Spatial Transcriptomics; aHSC, activated HSC; APAP, acetaminophen; CCl4, carbon tetrachloride; cDC, conventional dendritic cell; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; CN, cellular neighborhood; DC, dendritic cell; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DSP, Digital Spatial Profiler; EC, endothelial cell; FF, fresh frozen; FFPE, formalin-fixed and paraffin-embedded; HB, hepatoblast; HE, hematoxylin and eosin; HSC, hepatic stellate cell; ICC, intrahepatic cholangiocarcinoma; KC, Kupffer cell; LPLC, liver progenitor-like cell; LSEC, liver sinusoidal endothelial cell; NK, natural killer; NKT, natural killer T; NPC, nonparenchymal cell; PC, parenchymal cell; PHx, partial hepatectomy; qHSC, quiescent HSC; SAM, scar-associated macrophage; smFISH, single-molecule fluorescence *in situ* hybridization; TF, transcription factor; TME, tumor microenvironment.

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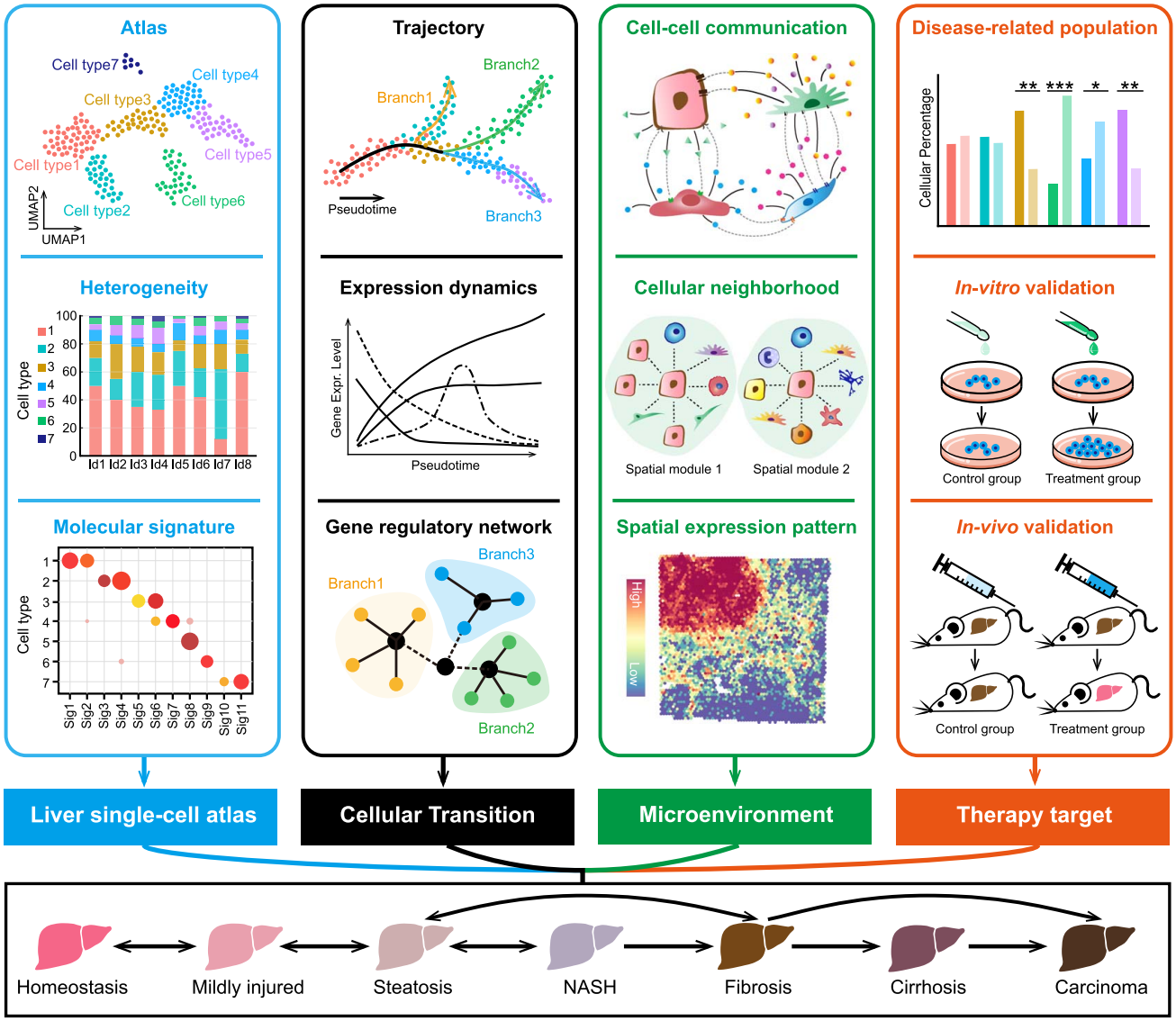

FIGURE 1 The overview of the single-cell and spatially resolved transcriptomics in liver biology. Applying single-cell and spatially-resolved technologies in liver research enables (i) the construction of liver single-cell atlas (the first panel), which allows to identify cell populations composed the organ, investigate cellular heterogeneity, and delineate molecular signature of individual cell types; (ii) the deduction of cellular transitions from pseudotemporal analysis (the second panel), which permits to depict gene expression dynamic along pseudotime and uncover key regulatory factors underlying cellular state transition based on gene regulatory network; (iii) the characterization of tissue microenvironments (the third panel) in the aspects of ligand-receptor mediated cell-cell communications, cellular neighborhoods identified from spatial proximity analysis, and spatially varied gene expression patterns; and (iv) the identification of potential therapy targets (the last panel), which may be informative for downstream *in vitro* and *in vivo* experiments. "*", "**", and "***" indicate the significant differences between different biological conditions. These help decode the liver, facilitating the investigation of molecular mechanisms underlying pathogenesis of liver diseases.

dynamics along cell state transitions and discovering the core regulatory factors driving the transitions[10–13]; (iii) characterizing the features of microenvironments such as cell interactions and spatial proximity[5,14,15]; and (iv) identifying the potential biomarkers and therapy targets for liver diseases.[12,16–18]

In this review, we first introduce the cutting-edge single-cell sequencing and spatially resolved technologies and then illustrate how these techniques address important questions in cellular landscape, molecular dynamics, and spatial architectures of the liver in the aspect of homeostasis maintaining, liver development, liver regeneration, and pathogenesis of liver diseases. The challenges and possible solutions regarding experiments and computational analyses are summarized when applying these technologies to liver research.

SINGLE-CELL AND SPATIALLY RESOLVED TECHNOLOGIES

ScRNA-seq is able to profile whole transcriptome at single-cell resolution.[19,20] It is widely used in liver research.[5,7,10,14,21,22] To conduct scRNA-seq, the liver

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must undergo dissociation, single cell isolation, transcript capture, and sequencing. There are diverse experimental platforms to perform scRNA-seq,[23] such as 10X Chromium,[24] Smart-seq2,[25] and MARS-seq.[26] Since it is superior in throughput,[27] 10X chromium is widely used to establish the cellular landscapes of the liver under different circumstances.[5,6,28,29] Smart-seq2 provides deep sequencing coverage,[30] which captures more genes per cell, allowing the identification of subtle differences in hepatic cells between different states.[18,22,31]

However, the following problems may be encountered when applying scRNA-seq to liver samples: (i) scRNA-seq requires enzyme-involved tissue disassociation that can lead to loss of sensitive cells[20,32,33] and changes in gene expression.[32,34,35] It has been reported that scRNA-seq presents altered cell proportions,[5,33,35] particularly hepatocytes, as they are highly sensitive to cell death when disassociated.[20,29,35] Moreover, scRNA-seq induces extra damage to hepatic cells.[33,34] In addition, tissue disassociation may generate debris when applied to injured livers and render low efficiency of cell release from highly fibrotic specimens.[20] (ii) It is hard to reflect the polyploidy nature of hepatocytes,[36] which includes nuclear ploidy (diploid, tetraploid, octoploid, and so on) as well as cellular ploidy (mononucleate and binucleate).[29] Polyploidy hepatocytes tend to have more transcripts,[36,37] but their gene expression profiles remain largely similar to those of diploid hepatocytes.[36–39] It is difficult to discriminate cells of varied ploidy purely based on data analysis. In addition, these polyploidy hepatocytes will confound doublet detection, particularly the "homotypic" ones.[40]

To overcome the challenge posed by low viability of hepatocytes, MacParland et al[29] developed a new cell isolation approach without density gradient, column purification, or flow cytometry, which successfully reconstructed the cellular landscape of healthy human liver. Besides that, recent practices suggest that single-nuclei RNA sequencing (snRNA-seq) may be the key to solve problem (i).[5,20,32,33] Instead of profiling intact cells, snRNA-seq measures transcripts within nuclei, which is compatible with fresh frozen (FF) samples and those hard to dissociate.[20,41] It has been shown to faithfully present the cellular proportions of hepatic cells.[5,37] In addition, snRNA-seq data set of intact liver samples showed less transcriptomic disruption compared with those predisassociated ones.[34] Moreover, it has been applied to comprehensively profile cellular landscape of injured livers induced by either hepatotoxic chemicals[34,42] or chronic liver diseases.[5,9] As for problem (ii), unfortunately, there is not a good computational solution yet.[20,29] Fluorescence-activated cell sorting may partially resolve the problem, which has been applied to purify hepatocytes of different ploidies according to genome contents ahead of single-cell sequencing.[37,38]

Besides scRNA-seq and snRNA-seq, there are a variety of other experimental methods to perform high-throughput single-cell studies.[4,19,20] Single-cell assay for transposase accessible chromatin sequencing (ScATAC-seq) enables profiling of the open chromatin landscape at single-cell resolution.[43] CITE-seq (short for "Cellular Indexing of Transcriptomes and Epitopes by sequencing") is a sequencing-based method that studies cells concurrently at transcriptomic and proteomic levels.[44] It simultaneously profiles surface proteins and transcriptome of single cells, making it very suitable for phenotyping immune cell subpopulations.[5,45,46] For specific cell type, scTCR-seq allows for full-length alpha and beta chain sequence pairing and determines the diversity, clonal expansion, and activation of T cells.[47] Moreover, single-cell multi-omics studies through either integrative analysis of different single-cell omics data[48] or measuring multiple types of molecules from individual cells, such as single-cell multiome assay for transposase accessible chromatin and gene expression assay,[49,50] facilitate the discovery of new biological insights more comprehensively and systematically. All of the aforementioned single-cell technologies have been applied in liver research, greatly promoting the development of the field.[5,8,10,17,46,51,52]

Despite the remarkable resolution, the aforementioned single-cell technologies lose spatial context. Recent advances in spatial omics serve as a complement and pave the way for completely understanding the liver in a spatial setting.[4,5,15,53,54] There are 2 categories of spatially resolved transcriptomics[55]: imaging-based technologies encompass *in situ* sequencing-based approaches and *in situ* hybridization–based approaches, such as MERFISH[56] and SeqFISH,[57,58] and sequencing-based technologies consist of laser capture microdissection-based sequencing,[59,60] 10X Genomics Visium Spatial Transcriptomics (10X Visium),[61,62] Seq-Scope,[63] Stereo-seq,[64] and so on. These diverse technologies are different in spatial resolution (dozens of cells, cellular, and subcellular), gene coverage (several to thousands genes or whole transcriptome), detectable tissue size, cell throughput, and compatible sample types.[65] 10X Visium is compatible of both formalin-fixed and paraffin-embedded (FFPE) and FF samples.[61,66] Within each capture area, 10X Visium is able to profile whole transcriptome of ~5000 spots (55 μm in diameter), providing an average resolution of 1–10 cells per spot.[61,66] NanoString GeoMx Digital Spatial Profiler (DSP) is also suitable for FFPE and FF tissue samples.[67] Unlike 10X Visium, DSP allows to profile whole transcriptome of user-defined regions of interest (covering 1–5000 cells) when ligated to next-generation sequencing system.[67] In addition, when ligated to nCounter system, DSP is capable of generating up to 96-plex spatial proteomics data.[67] The recently emerged stereo-seq achieves subcellular resolution at a larger field

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of view from FF tissue slide based on DNA nanoball technology.[64]

In addition, technologies for spatially resolved proteomics, metabolomics, or multiomics have been developed.[68–73] Multiplex immunohistochemistry enables the simultaneous detection of more than 7 proteins.[68] New strategies, such as imaging mass cytometry,[69] multiplexed ion beam imaging,[70] and CODEX,[71] enabling simultaneous analysis of dozens of proteins, offer enough power to distinguish cell types and investigate the spatial distributions of cells. Matrix-assisted laser desorption ionization-mass spectrometry imaging and desorption electrospray ionization mass spectrometry imaging are the most commonly used spatial metabolomics techniques.[72] The former can consistently detect hundreds of metabolites at a spatial resolution of <10 μm.[72] NanoString CosMx Spatial Molecular Imager is a flexible high-plex *in situ* analysis platform that can detect up to 1000-plex RNAs and 100-plex proteins from FFPE or FF samples at cellular and subcellular resolution.[73]

Rapid development of experimental techniques has been accompanied by the accumulation of data. To bridge the gap between data and biological insights, computational approaches are becoming increasingly important. Basic suite for scRNA-seq data analysis includes quality control, normalization, unsupervised clustering, gene signature detection, cell type annotation, and visualization with either TSNE or UMAP.[74,75] Computational packages such as Seurat,[76] Scanpy,[77] and RaceID3[78] are capable of the aforementioned processing. More complex analyses, such as batch effect correction, trajectory inference, pathway activity estimation, cell–cell communication deduction, and the construction of single-cell regulatory networks, have been addressed by various bioinformatic tools and illustrated thoroughly in previous benchmark works.[79–81]

Cell type/state annotation is the fundamental step in analyzing scRNA-seq data but remains challenging in the field.[82–84] Generally, dimensionality reduction and clustering are performed to gain cell clusters, which are subjected to automated and/or manual annotation to determine their cell identities or states.[76,82] Automated strategies rely either on curated marker gene databases, gene expression correlation to reference data sets, or label transferring through supervised classification.[82] Thus, the results are heavily influenced by the provided references. Computational tools enabling such analysis include scmap,[85] SCINA,[86] OnClass,[87] SingleCellNet,[88] scNym,[89] and others. Unlike automated annotation, manual annotation determines cell identities or states mainly relying marker genes, which demands a strong biological understanding.[84] Researchers may need to collect well-recognized markers through searching literatures and/or mining existing single-cell data sets for gene signatures concerning the queried data set.[84] Both strategies have been applied to liver single-cell studies.[5,29,90] A 3-step workflow has been proposed to perform high-quality annotation: first use automated tools as they are comprehensive and convenient, and then perform manual curation, which is more flexible and reliable for novel or rare cell populations, and finally, experimentally verify the annotation.[84] Notably, annotating cell states, particularly intermediate states of a cell gradient,[84] emerges another challenge as they may not express unique markers.[91,92] However, it is easy to define the end state of a gradient whose signature genes would reversely help characterize the intermediate states.[84]

Computational methods designed for spatial omics are under development.[93–95] The core idea of these methods is the use of gene expression profiles, spatial distances, and histopathological features to characterize tissue spatial architectures. The common scenarios (and existing tools) are as follows: (i) unsupervised division of spatial domains (STAGATE,[96] SpaGCN,[97] and BayesSpace[98]); (ii) trajectory construction in a spatial context (stLearn[99] and SPATA2[100]); (iii) the identification of spatially variable genes (SpatialDE[101] and SPARK[102]), and (iv) the prediction of spatial gene expression from hematoxylin and eosin staining (ST-Net,[103] XFuse,[104] and HE2RNA[105]). Moreover, the integration of scRNA-seq with spatially resolved transcriptomics enables complementary analysis: the decomposition of cell populations mixed in Visium spots (SPOTlight[106] and cell2location[107]), the inference of potential spatial origins of individual cells,[108] and resolving cellular heterogeneity from a perspective of spatial distribution.[5]

LIVER HOMEOSTASIS

Construction of a single-cell atlas of the homeostatic liver

Two experimental strategies are applied to establish the single-cell atlas of the liver. Unbiased sampling enables the recapitulation of entire cell populations of the liver.[7,29,35] Applying this strategy, the Tabula Sapiens,[109] MacParland et al,[29] and Aizarani et al[7] have captured ~5000, 8400, and 10,000 cells from normal livers of human donors, respectively. Presorting cells of interest before sequencing provides a more focal perspective on specific cell types, facilitating the discovery of novel subpopulations.[18,45,110] For instance, focusing on $CD45^+$ immune cells, Zhao et al[110] have constructed an immune cell landscape containing more than 70,000 cells from paired liver, spleen, and peripheral blood samples belonging to 3 healthy human donors. In addition, the single-cell transcriptomic atlas of normal mouse livers has also been comprehensively characterized by the Tabula Muris Consortium[111,112] and other studies.[5,113]

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There are 2 major categories of hepatic cells: parenchymal cells (PCs) and nonparenchymal cells (NPCs). PCs consist of hepatocytes and cholangiocytes (ie, biliary epithelial cells), accounting for ~80% of all cells in the liver.[114,115] The rest are NPCs, which are composed of immune cells, including T cells, B cells, natural killer (NK) cells, neutrophils, dendritic cells (DCs), monocytes, and macrophages, as well as stromal cells, including endothelial cells (ECs) and mesenchymal cells.

Efforts being made to comprehensively delineate the cellular landscape of the livers greatly facilitate the identification of robust cell markers and the discovery of novel/rare cell populations.[5,7–9,11,29] The classical or newly identified gene/protein markers for common hepatic cells in mouse (Figure 2A) and human (Supplemental Figure S1, http://links.lww.com/HEP/F22) are summarized in Supplemental Table S1, http://links.lww.com/HEP/F21. For example, based on single-cell studies, a core gene signature specific to liver-resident KCs, including *CD5L*,[5,7,29,116,118] *VSIG4*,[5,7,29,116] *CD163*,[5,7,8,29,116] *FOLR2*,[5,7,8,29,116] *MARCO*,[5,7,29,116] and *SLC40A1*[5,8,116,118] has recently been proposed, which is conserved in not only human and mouse but also in other 5 different species.[5,116] Moreover, novel macrophage subpopulations have been detected by scRNA-seq studies.[5,8,118] For example, a macrophage subset termed lipid-associated macrophage, which is characterized by the expression of *TREM2*, *CD9*, and *SPP1*, has been recently identified through scRNA-seq and CITE-seq.[5,45,119] The subpopulation was found expanded in mice and humans with obesity.[5,45,119] As for rare cell populations, a small epithelial cell population positive for *EPCAM* and showing an intermediate expression level of *TACSTD2* (also known as *TROP2*) has been observed in adult human livers.[7] *In silico* lineage reconstruction and *in vitro* experiments both demonstrated that *EPCAM*$^{+}$-*TROP2*^{int} epithelial cells were capable of generating hepatocyte-biased and cholangiocyte-biased cells, suggesting that they are liver progenitor cells.[7]

Liver zonation

The aforementioned cells are neatly packed into liver lobules, the structural units of the liver (Figure 2B). On a liver lobule, hepatocytes radiate outward from the central vein to the portal triad, an anatomic structure containing bile ducts, the portal vein, and the hepatic artery.[1,120] Oxygen, nutrients, and hormones exhibit gradient distribution along the portal-central axis, resulting in nonuniformly expressed metabolic genes across the lobule.[1,120–122] Generally, the liver lobule is divided into 3 regions: the periportal area (zone 1), the pericentral area (zone 3), and the middle area (zone 2) (Figure 2B). This is termed liver zonation. Previous work has revealed a few zonation landmark genes for hepatocytes.[120,123,124] However, to fully understand liver zonation, a transcriptome-wide zonation landscape of diverse hepatic cells is needed.

Advanced experimental techniques and computational approaches facilitate the deciphering of zonal expression patterns of hepatic cells from scRNA-seq data sets.[7,18,125–127] Combining scRNA-seq and single-molecule fluorescence *in situ* hybridization (smFISH), Halpern et al[127] expanded the zonation pattern to whole transcriptome in mouse hepatocytes. They first divided the porto-central axis into 9 layers according to smFISH of 6 landmark genes (pericentral: *Glul* and *Cyp2e1*[124]; periportal: *Ass1*,[123] *Asl*,[123] *Alb*,[120] and *Cyp2f2*[124]), which was then used to build a probabilistic algorithm to infer the layer of hepatocytes assessed by scRNA-seq and then estimated the zonal pattern of other genes.[127] The zonal genes summarized by this pioneering work offer the possibility to deduce lobular locations of hepatocytes for subsequent studies.[5,29,125] For mouse ECs, paired-cell RNA-seq has been developed to infer their lobular coordinates according to the zonal layer of physically adjacent hepatocytes.[125] Observing that mouse hepatocytes and ECs exhibit continuous transcriptome-wide zonation,[125,127] Aizarani et al[7] reasoned that the zonal gene expression patterns of human hepatocytes and LSECs would be reflected at their major axis of variability when assessed by scRNA-seq. Thus, they ordered human hepatocytes and LSECs by the diffusion map and then deduced the zonal gene pattern by modeling gene expressions against inferred cell orders.[7] A similar idea was adopted to infer zonation pattern of HSC.[18] Using independent component analysis, Dobie et al[18] identified the component most highly correlated with the experimentally validated HSC periportal marker (*Ngfr*) and then regarded genes with high loadings of that component as HSC zonation genes.

In addition, 10X Visium reflects zonation patterns more intuitively.[5,6,128] The main procedures to deduce zonated expression patterns from Visium data sets include (i) labeling the positions of veins according to histological features or the expression of landmark genes or both and (ii) identifying the genes exhibiting zonal patterns by modeling gene expression against the distance to the veins.[5,6,128] Furthermore, by integrating scRNA-seq, the proportions of hepatic cells within each spot could be deconvoluted; thus, the distributions of hepatic cells along the portal-central axis could be deduced.[5]

Approximately 60, 140, and 80 landmark genes for the zonation of mouse hepatocytes, ECs, and HSCs have been identified, respectively.[18,125,127] Nearly 50% of hepatocyte genes[127] and 30% of EC genes[125] are nonrandomly expressed. The expression of most of these genes monotonically increases or decreases portal-centrally, and only a few genes show peak expression in the intermediate lobule layers.[125,127] For mouse hepatocytes, periportally biased genes are

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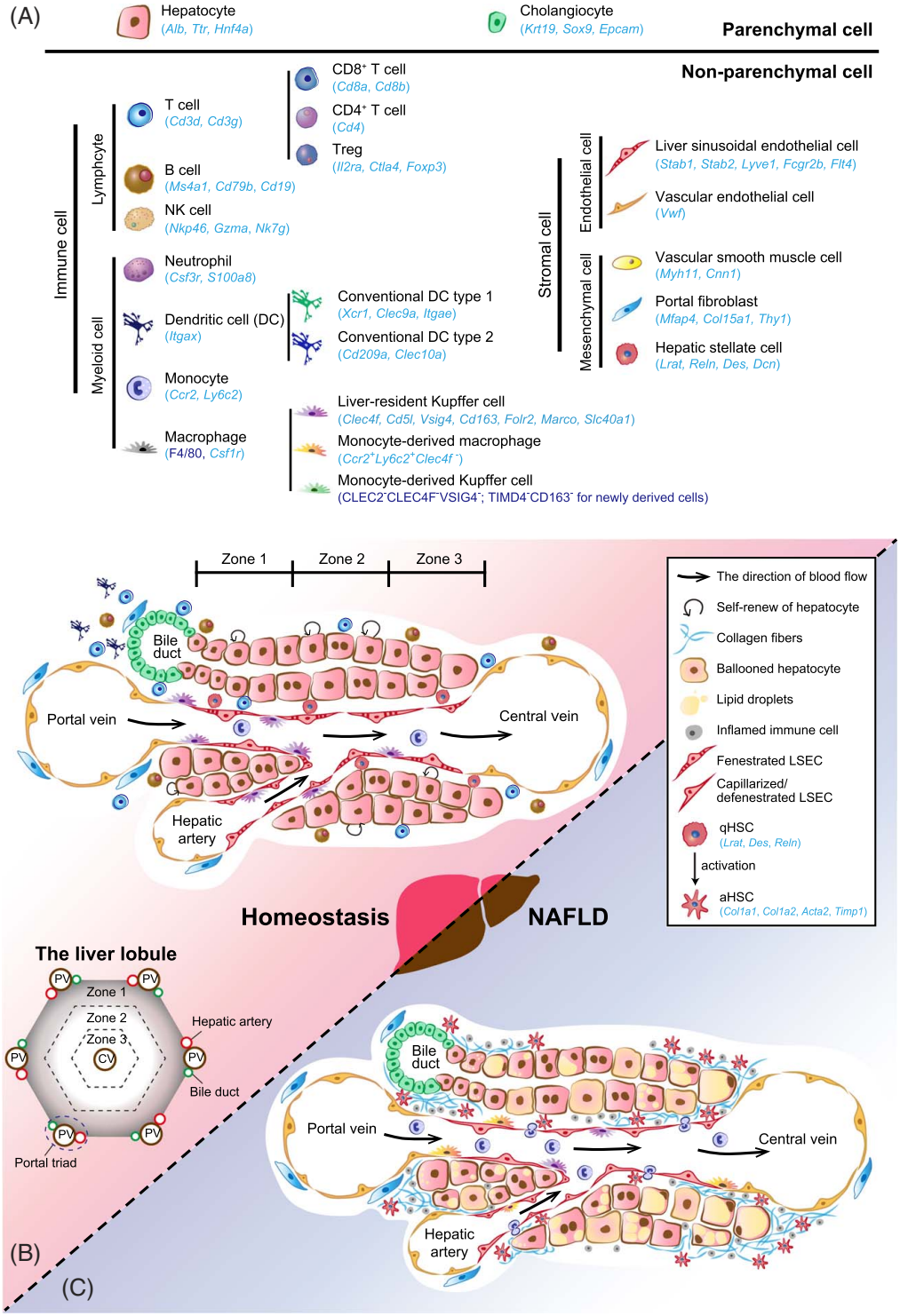

FIGURE 2 Common hepatic cell types of murine liver and their zonal distributions in homeostatic and NAFLD livers. (A) Common hepatic cell types in mouse liver. Gene (in light-blue)/protein (in dark blue) markers are listed below corresponding cell populations. Since monocyte-derived KC acquires most of hallmarks of liver-resident KC, it is hard to discriminate them unless the newly derived ones.[116]. Please refer to Supplemental Table S1 (http://links.lww.com/HEP/F21) for detailed resources for each marker. (B) Schematic diagram of the structural unit of the liver—liver lobule (left-bottom panel) and the zonal distributions of hepatic cells in homeostatic liver. (C) Representative view of liver lobule in NAFLD mouse. NAFLD liver is characterized by the accumulation of lipid droplets in hepatocytes, the emergency of ballooned hepatocytes, defenestrated LSECs, increased inflamed immune cells, and the accumulation of collagen fibers. Notably, during NAFLD pathogenesis, HSC transitions from a quiescent state (qHSC) to activated state (aHSC). The phenomenon is observed with no zonal bias in mouse NAFLD livers.[92,117]

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enriched in oxidative phosphorylation, liver secreted proteins, and complement cascades, which have been further demonstrated to be closely related to KRAS signaling.[127] Pericentrally biased genes are involved in detoxification, bile acid biosynthesis, and proteasome components whose master regulator is WNT signaling.[54,127] Beyond these, the distributions of hepatic myeloid cells and lymphocytes across murine liver lobules have been revealed (Figure 2B).[5,126] B cells, T cells, and stromal cells could be seen across all zones, while conventional DCs (cDCs) majorly enrich near the portal vein[5]; KCs preferentially locate in periportal zone[5,126]; and NK T cells are also concentrated around periportal region.[126]

Notably, zonal patterns may vary by time, biological conditions, and species. A correlation between biological rhythms and hepatocyte zonal patterns has been observed in mouse livers.[129] Based on a mixed-effect model, ~20% of hepatocyte genes are rhythmic on a 24-hour time scale, and 7% of them are both zonated and rhythmic.[129] When treated with acute acetaminophen (APAP), many zonation genes undergo expression alterations, making their zonal patterns no longer exist, while the others remain zonally different.[6] Moreover, there are discordant and concordant zonation profiles between mice and humans.[130] For example, the pericentral genes *CYP1A2* and *LGR5* and the periportal genes *HAL* and *SDS* of human hepatocytes remain conserved in mice, while *SLC2A2* expressed pericentrally in humans but periportally in mice.[130] Therefore, one should choose appropriate landmark genes for a given biological condition. In our opinion, genes that are highly expressed in a zonal manner and remain robust to cofactors may be good candidates.

LIVER DEVELOPMENT

Liver development proceeds through several phases. During embryonic liver development in mice (Figure 3A), soon after the hepatic specification of endodermal cells, the liver diverticulum forms. The hepatoblasts (HBs) within the liver diverticulum then migrate, forming the nascent liver bud, which grows and gradually forms the liver.[131–133] The liver keeps maturing after birth until its functions are fully established.[28] The whole process involves dramatic cellular transitions.[28,134,135] By depicting these transitions, scRNA-seq studies have constructed the roadmap of how hepatic functions are established and how liver zonation forms.[91,134–136]

The establishment of hepatic functions follows a stepwise manner

The organogenesis of the mouse liver initiates from the transition of foregut epithelial cells into HBs (hereafter the transition is referred as "hepatic transition").[91] Using scRNA-seq, Mu et al[91] have depicted the cellular roadmap of the hepatic transition and found that the transition followed a linear trajectory, which was characterized by the gradual depletion of epithelial features (*Krt19*, *Epcam*, and *Cldn6*) as well as a gradual upregulation of HB markers (*Hnf4a*, *Dlk*, *Ttr,* and *Alb*) (Figure 3B). Moreover, an intermediate state possessing both epithelial and hepatic features was observed between foregut epithelial cells and HBs, which was named liver primodium cell (Figure 3B).[91]

Blood coagulation, hemostasis, and protein complex assembly have been found significantly activated at the very beginning of hepatic transition, and continuously upregulated until the generation of hepatocytes[91,135]; oxidation–reduction processes, lipid biosynthesis, and metabolism are significantly induced since liver primodium cells were emerged.[91] In addition, genes playing important roles in major hepatic functions, such as detoxification, transport, fatty acid metabolism, and glucose metabolism, have been found progressively upregulated when HBs differentiated into hepatocytes.[135] Together, the hepatic functions are established in a stepwise manner during mouse embryonic liver development (Figure 3B).

Tracing the differentiation of HBs

HBs are bipotent progenitors that are capable of differentiating into hepatocytes and cholangiocytes during liver development.[131–133] By pseudotemporal analysis, scRNA-seq studies have identified a branched trajectory of HB differentiation (Figure 3B) that is conserved between mice and humans.[134–136] Based on the trajectory, investigators have determined the molecular dynamics and precise gene regulation driving the different fates of HBs (Figure 3B).[134–136]

In mice, as HBs differentiate into hepatocytes, the expression of transcription factors (TFs) that are essential for hepatocyte lineage development, such as *Ppara*, *Rora*, and *Nr1i3*, have been found induced immediately once HB emerges.[134–136] In combination with the sequential acquisition of hepatic functions mentioned earlier, the process is considered the "default" route for HB differentiation.[134,135] In contrast to hepatocyte lineage development, biliary lineage development seems to be highly regulated. During mouse liver development, some HBs express TFs that control biliary lineage development, such as *Sox9*, *Sox4*, *Hnf1b*, and *Onecut1*, and gradually differentiate into cholangiocytes.[134,135] Pseudotemporal analysis has shown that pathways controlling biliary morphogenesis, such as epithelial tube morphogenesis, WNT signaling, and NOTCH signaling, are significantly upregulated during HB-to-cholangiocyte transition in

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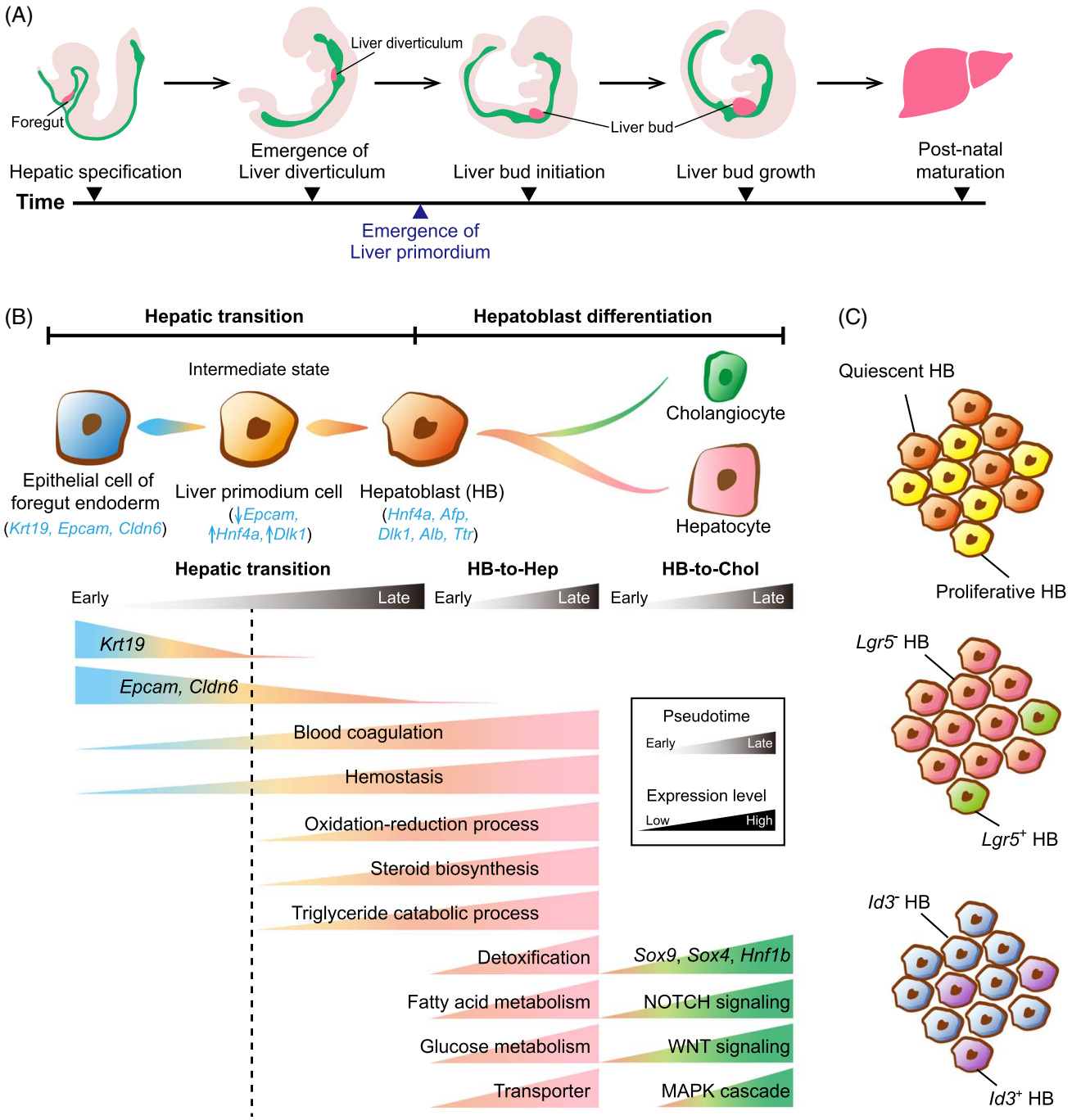

FIGURE 3 Pseudo-temporal analyses reveal the cellular trajectory of mouse embryonic liver development. (A) Schematic diagram showing different phases of mouse liver organogenesis. (B) The cellular trajectory and the molecular dynamics of the development of hepatic epithelium. Epithelial cells of foregut endoderm decrease the expression of epithelial signature genes, upregulate hepatic genes, and gradually transition into hepatoblasts (HBs) (the transition is referred as "hepatic transition" in this article). During hepatic transition, an intermediate state expressing both epithelial and hepatic gene signature at low level has been identified,[91] which was regarded as emergence of liver primordium [labelled in dark blue in (A)]. HBs would further differentiate into hepatocytes (HB-to-Hep) or cholangiocyte (HB-to-Chol). Key regulatory genes and biological processes underlying the whole development process are shown. (C) Heterogeneity of hepatoblasts in embryonic mouse livers. Notably, the heterogeneities in the aspects of proliferative status and expression of *ID3* are also observed in human embryonic livers.

mice (Figure 3B).[135] It is worth noting that the PKC/MAPK cascade is strikingly upregulated from the middle phase of mouse cholangiocyte development.[135] When treated with a PKC agonist for 48 hours, mouse HBs displayed clear cholangiocyte characteristics, indicating that the MAPK cascade could promote cholangiocyte maturation in the mouse embryonic liver.[135]

In addition, scRNA-seq studies identified heterogeneities of mouse HBs in the aspects of proliferative status (also seen in human HBs), expression of *Lgr5*, and

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expression of *Id3* (*ID3* in human HBs) (Figure 3C).[134–136] Proliferative HBs account for a large proportion of total HBs and are observed across different embryonic development stages in both mouse and human fetal livers.[134,135] *Lgr5*$^+$ HBs and *Id3*$^+$ HBs constitute only a small proportion of total mouse HBs (~2% and <6%, respectively) and tend to be present at the early stage of liver development.[134,136] Interestingly, these heterogeneities have little effect on the differentiation potential of HBs. All of the abovementioned HBs could give rise to hepatocytes and cholangiocytes *in vitro* or *in vivo,* without significant differences in either their distributions along the *in silico* lineage trajectory or the efficiency of differentiation.[134–136] One hypothesis is that different biological pathways are invoked during HB differentiation, each of which could drive differentiation independently and compensate for the other, guaranteeing the morphogenesis of the liver.

The formation of liver zonation

Another intriguing question is when liver zonation forms. During human embryonic liver development, 2 functionally different subpopulations of maturing hepatocytes have been identified.[134] One subpopulation was represented by high expression of *VTN*, which was active in coagulation, detoxification, and organic acid metabolism.[134] The other subpopulation highly expressed *PRL13* and was associated with increased growth ability.[134] Although these subpopulations tended to occupy different regions of liver lobules at 19 weeks postcoitum in humans based n RNA *in situ* hybridization experiments, the spatial pattern was unrelated to liver zonation.[134] Moreover, these 2 subpopulations were absent from mouse fetal livers.[134] Therefore, liver zonation may not be fully formed before birth.

Combining scRNA-seq and immunostaining, Liang et al[28] have assessed the gene expression dynamics of hepatocytes in newborn mice at postnatal days (D) 1, 3, 7, 21, and 56. Gene expression analysis of 18 hepatocyte landmark genes and immunostaining of representative zonation hallmarks (E-cadherin and CYP2E1) revealed a gradually distinguishable zonation signature from D1 to D56.[28] When aligned to the previously defined zonation layers,[127] only hepatocytes at D56 exhibited a consistent layer distribution with that of adult hepatocytes.[28] In addition, hepatocytes from different layers at D21 were still mixed when projected into TSNE space, suggesting that liver zonation was not yet fully developed in D21 hepatocytes.[28] Together, these data demonstrate progressive zonal construction in postnatal mouse livers.

LIVER REGENERATION

The liver has a remarkable regeneration capacity. Experimental models with liver injury induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine, (DDC)[10] carbon tetrachloride (CCl_4),[18] APAP,[6] and partial hepatectomy (PHx)[13] have been widely studied in this field.[137–139] By scRNA-seq and spatially resolved transcriptomics, investigators have unveiled new insights into the cellular source and the molecular trajectory of liver regeneration.[6,10,13,52,140]

The cell source of hepatocyte proliferation

Hepatocytes are able to self-renew in the homeostatic liver and enter the cell cycle rapidly once the liver is damaged.[114] However, whether this capacity is possessed by specific cell subpopulations remains controversial. In homeostatic mouse livers, lineage tracing-based studies have reported several hepatocyte subpopulations with an elevated ability to proliferate, including hybrid hepatocytes[141] expressing low amounts of *Sox9* near the PV, *Tert*^{high} hepatocytes,[142] and LGR4$^+$ hepatocytes[143] that are stochastically distributed across lobular zones, as well as a hepatocyte subpopulation situated at middle zone.[144,145] In healthy human livers, an *AFP*$^+$ hepatocyte subpopulation displaying an enhanced cellular division without a regional enriched pattern has been recently revealed by scRNA-seq study.[29] On injury, varied hepatocyte subpopulations have been implicated in liver regeneration. By lineage tracing, periportal hybrid hepatocytes could give rise to new hepatocytes after repetitive administration of CCl_4 to mouse livers,[141] while mid-zone hepatocytes have been implicated in liver regeneration after PHx injury.[13,144,145] However, in APAP-induced mouse liver injury, as revealed by scRNA-seq, 10X Visium, and smFISH, *Mki67*$^+$ proliferative hepatocytes were not enriched in specific zones.[6] Taken together, these findings illustrate that the cell sources of hepatocytes that possess elevated proliferation ability may depend on injury conditions.

The molecular dynamics in post-PHx regeneration

The proliferation of hepatocytes is thought to be responsible for regeneration in mildly injured livers and those after PHx.[114,138,139] Relying on pseudotemporal analysis, recent scRNA-seq studies have further demonstrated the molecular dynamics during post-PHx regeneration of mouse livers.[13,52] After downregulating metabolism-related pathways, such as fatty acid metabolism and amino acid metabolism, quiescent hepatocytes entered a transition state.[13] A remarkable proportion of hepatocytes in the transition state reactivated those previously suppressed metabolic pathways entering a metabolically hyperactive state, while the others upregulated the cell cycle, RNA splicing, and other proliferation-

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related pathways and started to proliferate.[13] These suggest a division-of-labor model of post-PHx regeneration, which is thought to balance the proliferation and metabolic demands of a regenerating liver.[13]

Fetal/progenitor-like programs are upregulated during liver regeneration

Interestingly, gene expression programs activated in fetal or postnatal livers have been found induced in post-PHx regeneration of murine livers (Figure 4A). TFs activated in mouse postnatal day 14 hepatocytes, such as RELA, E2F1, and TRP53, are upregulated in regenerating hepatocytes after PHx.[13] Moreover, through integrating scRNA-seq and scATAC-seq, a hepatocyte subpopulation at 48 hours after PHx was identified that expressed progenitor-related genes (*Afp*, *Axin2*, and *Yap*), whose master TFs include those controlling embryonic liver development (*Gata6*, *Sox9*, and *Sox17*).[52] This phenomenon was confirmed by immunohistochemistry staining.[52] These data indicate the emergence of fetal-like programs during post-PHx regeneration. Notably, fetal-like programs existed transiently, with the signal markedly decreased at 72 hours after PHx and nearly undetectable at 96 hours after PHx.[52] In addition, in severely injured mouse livers induced by APAP, fetal-like programs, such as the expression of *Afp*, could also be traced (Figure 4B).[6] Specifically, smFISH reveals that *Afp* was expressed exclusively in the interface between damaged and nondamaged regions, which represented the leading edge of regeneration on APAP injury.[6]

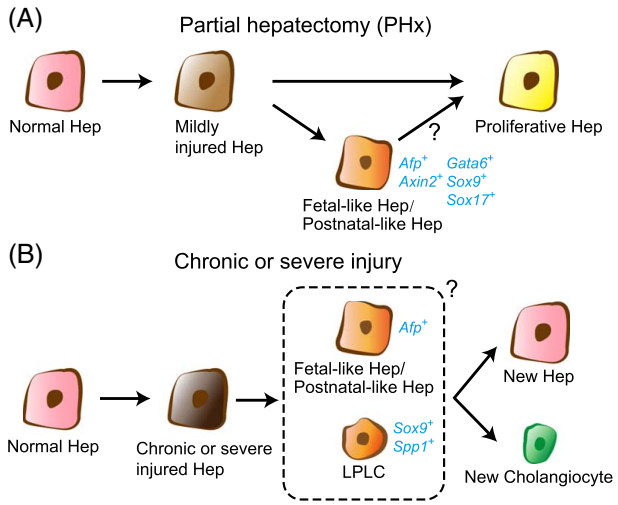

FIGURE 4 Single-cell studies unveil cellular transitions of hepatocytes during mouse liver regeneration. (A) On partial hepatectomy (PHx), a hepatocyte (Hep) subpopulation expressing gene signatures related to fetal/postnatal hepatocytes has been identified.[13,52] However, whether Heps must go through such a fetal/progenitor-like state to enter cell cycle remains elusive. (B) On chronic or severe injury, a hepatocyte subpopulation resembles liver progenitor-like cells (LPLCs) has been identified in mouse livers.[6] To be noted, whether these fetal/progenitor-like hepatocytes are the same population or subpopulations of LPLCs remains elusive.

These fetal-like expression programs resemble those activated in liver progenitor-like cells (LPLCs).[10,146] LPLCs are hybrid cells displaying transcriptomic features of both hepatocytes (*Hnf4a* and *Alb)* and cholangiocytes (*Sox9* and *Spp1*).[10,146] On chronic or severe injury, mature epithelial cells may reprogram to LPLCs, which contribute to liver regeneration by giving rise to new hepatocytes and cholangiocytes (Figure 4B).[114,140,147] Previously identified progenitor-like signature[147] induced during chronic injury was significantly upregulated in fetal-like hepatocytes at 48 hours after PHx.[52] Together, these results indicate that fetal/progenitor-like programs are upregulated during liver regeneration. Whether these fetal/progenitor-like hepatocytes that emerge under different circumstances are the same population or subpopulations of LPLCs remains elusive. For now, their identification heavily relies on a few markers.[6,13,52,146] Studies systematically comparing the molecular traits of all these fetal/progenitor-like populations are urgently needed. In addition, it is still unclear whether hepatocytes must go through such a fetal/progenitor-like state to enter the cell cycle. Notably, it has been reported that ~24.2% of hepatocytes were derived from LPLCs in DDC–treated mouse livers.[148] In our opinion, it is possible that there are multiple mechanisms functioning simultaneously to guarantee the rapid repair and regeneration of the liver.

The bipotential ability of LPLCs implies great clinical significance in regenerative medicine. Given the relatively small proportion of LPLCs, they are difficult to isolate.[149] To solve this problem, transition and expansion media have been developed to convert mature mouse or human hepatocytes into progenitor-like cells *in vitro*.[150,151] ScRNA-seq showed that transition and expansion media–induced human cells upregulated *EPCAM*, *SOX9*, *CD24*, and *CK19*, implying their resemblance to those genuine progenitors identified *in vivo*.[150] Induced LPLCs provide new ideas for the development of cell-based therapies, which may improve the treatment of chronic hepatic disorders.

NONALCOHOLIC FATTY LIVER DISEASE

NAFLD, a leading chronic liver disease worldwide, has now become the fastest growing cause of liver cancer.[152] The pathological spectrum of NAFLD encompasses simple steatosis, NASH, and fibrosis.[153–155] The liver fibrosis may further progress into the irreversible diseases including cirrhosis and cancer.[153,154] Although many efforts have been

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devoted to resolving the pathogenesis of NAFLD, the molecular mechanism of the disease remains incompletely understood. A major obstacle in this field is the limited accessibility of human NAFLD samples. Various animal models have been developed to mimic human NAFLD phenotypes,[156] such as CCl_4-induced liver fibrosis model and diet-induced NAFLD mouse models, including Western diet model, amylin liver NASH model (high-fat and high-fructose diet), high-fat and high-cholesterol model, choline-deficient but high-fat model, methionine- and choline-deficient diet model, and methionine-deficient and choline-deficient l-amino acid-defined, high-fat model.[16–18,113,157] These models successfully recapitulate the main characteristics of human NAFLD, thus are widely applied to construct the cellular landscape of the diseased livers and to identify NAFLD-associated cell subtypes, boosting the discovery of key factors in NAFLD.[5,8,18,113,158,159]

NAFLD-associated cell types

HSCs account for <10% of cells within the liver, but their activation is the major contributor of pathologic fibrosis in both patients and animal models.[155,158,159] On fibrogenic stimulation under different etiologies, quiescent HSCs would turn into activated HSCs (aHSCs) (Figure 2C).[92,160] Through scRNA-seq, 4 aHSC subpopulations have been identified in NASH livers of foz/foz mice, including the classical collagen-secreting myofibroblasts (*Acta2*$^+$*Col1a1*$^+$*Col1a2*$^+$*Timp1*$^+$), proliferative HSCs (*Cdk1*$^+$), inflammatory HSCs (*Cd36*$^+$*Ly6c*$^+$), and intermediate activated HSCs (*Irf7*$^+$) (Figure 5A).[92] Interestingly, the intermediate activated states of HSCs that showed less expression of fibrogenic genes were observed across studies,[11,92] suggesting a multi-step HSC activation during NASH pathogenesis. Besides cellular heterogeneity, zonal patterns of HSC activation have been determined by single-cell technologies and computational approaches.[18,92,117] Central vein-associated HSCs, but not portal vein-associated HSCs, were considered as the dominant cell source of aHSCs/myofibroblasts in CCl_4-induced fibrotic livers.[18] However, in diet-induced NASH mouse livers, there was no significant association between HSC activation and liver zones (Figure 2C).[92,117] Thus, the activation of HSC may exhibit a model-dependent zonal pattern.[18,92,117]

Dysfunction of LSECs is permissive for NAFLD pathogenesis and liver fibrosis (Figure 5B).[161,162] In diet-induced NASH models, increased lipid metabolism, antigen presentation, and chemokine release, represented by upregulation of *Fabp4*, *Cd36*, and *Cxcl9*, respectively, occurred in all LSEC subpopulations across different lobular zones (Figure 5B).[113] Such a dysfunctional phenotype (upregulation of *FABP4* and *CXCL9*) could also be traced in bulk RNA-seq data set of patients with NASH.[113] Similarly, dysfunction of LSEC, represented by the downregulation of gene programs in response to shear stress, presented in all zones of CCl_4-induced cirrhotic murine livers.[90] Moreover, combining scRNA-seq and IF staining, two novel EC subpopulations (CD34$^+$PLVAP$^+$VWA1$^+$ and CD34$^+$PLVAP$^+$ ACKR1$^+$) have been linked to scar formation in fibrotic niche of human cirrhotic livers (Figure 5B).[8] These scar-associated ECs express NOTCH ligands, which interact with NOTCH receptors on HSCs, thus activating HSCs through NOTCH signaling and promoting fibrillar collagen expression in HSCs (Figure 5B).[8] Capillarization, namely, loss of fenestrae and development of a basement membrane, is an another characteristic of LSEC that emerges from the early stage of NAFLD pathogenesis.[161,162] A zone 3-preferential LSEC capillarization pattern (upregulation of *Cd34*) has been observed in the CCl_4-induced mouse cirrhotic livers.[90] However, whether capillarization of LSECs displays zone-biased pattern in preclinic NASH models or human NASH livers remains elusive.

Macrophages have long been implicated in promoting inflammation and mediating fibrosis in the progression of NAFLD.[116,163] By comparing the cell components across different NAFLD pathologic stages, KCs have been found to be gradually lost during disease progression (Figure 5C).[16,45,164] Meanwhile, the number of infiltrating monocytes grows, and they rapidly differentiate into monocyte-derived macrophages and monocyte-derived KCs, compensating for the reduction in the number of KCs (Figure 5C).[45,164] Recent single-cell studies have identified novel macrophage subsets that are associated with NAFLD progression (Figure 5C). Lipid-associated macrophages marked by the expression of *Cd9*, *Spp1,* and *Trem2* were initially identified in adipose and liver tissues of obese mice and human,[45,119] which had been found latter derived from recruited circulating monocytes and conserved across multiple species.[5,45] NASH-associated macrophages highly expressing *Trem2*, *Gpnmb*, and *Cd9* were identified in amylin liver NASH–induced and choline-deficient l-amino acid-defined, high-fat–induced NASH mice.[113] Scar-associated macrophages (SAMs) were detected in fibrotic niches of human patients with cirrhosis and CCl_4-induced mouse fibrotic livers.[8] SAMs were able to promote human HSC proliferation through the interactions of the ligands TNFSF12 and PDGFA with the receptors TNFRSF12A and PDGFRA on HSCs.[8] Although identified in independent studies, lipid-associated macrophages, NASH-associated macrophages, and SAMs share similar molecular features regarding marker genes, raising the hypothesis that they are the same population (Figure 5C).[45,116]

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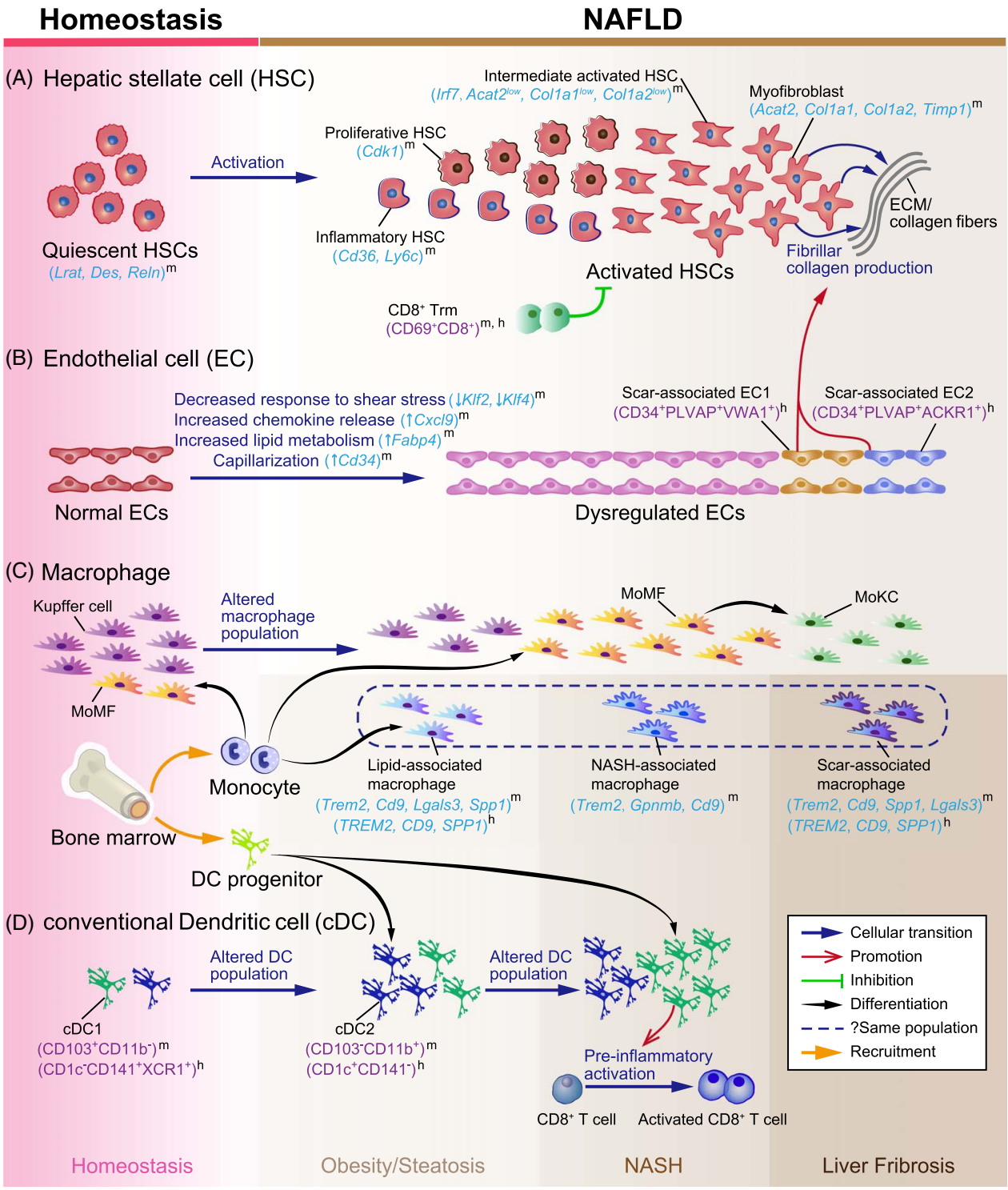

FIGURE 5 The transitions of NAFLD-associated cell populations during NAFLD pathogenesis. (A) The activation of HSC. Notably, 4 subpopulations of activated HSCs have been uncovered by scRNA-seq studies. (B) Normal EC undergoes transcriptomic changes and turns into dysregulated EC. There are 2 scar-associated EC subsets identified in the fibrotic niches of human cirrhotic livers. (C) The alternations in macrophage population. Macrophage shifts from a KC-dominated population to the one with profound increase in recruited macrophages. Subsets of macrophage have been identified associated with NAFLD including lipid-associated macrophage in obese/steatotic livers, NASH-associated macrophage in NASH livers, and scar-associated macrophage in fibrotic niche of cirrhotic livers. Given that they share common signature genes, there is an assumption that they are the same population.[45,116] (D) The population shifts of cDCs. The number of cDC2 cells is significantly increased in both obese and NASH mice, while cDC1 cells are more abundant in NASH than those of obese livers. Gene (light-blue)/protein (purple) markers of mouse (m) or human (h) are shown. Abbreviations: $CD8^+$ Trm, liver-resident memory $CD8^+$ T cell; cDCs, conventional dendritic cells; EC, endothelial cell; MoKC, monocyte-derived KC; MoMF, monocyte-derived macrophage.

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We hypothesize that these TREM2$^+$ macrophages emerge at the very beginning of NAFLD pathogenesis and constantly contribute to the progression of NAFLD.

In addition, there are other infiltrated immune cells linked to the progression of NASH through single-cell studies.[16,17,165] Compared with normal livers, exhausted CD8$^+$ T cells highly expressing *Pdcd1* and *Tox* have been found more prevalent in diet-induced NASH mouse livers.[165] Increased liver-resident CD8$^+$ memory T cells (CD69$^+$CD8$^+$) in high-fat and high-cholesterol–induced mouse NASH livers and human NASH livers have been shown to attract HSCs in a CCR5-depent manner and predispose aHSCs to apoptosis (Figure 5A).[17] Time-series scRNA-seq and flow cytometry assays indicated the elevation of cDCs derived from bone marrow cDC progenitors in obese and NASH mouse livers under different etiologies (Figure 5D).[16] Among them, the number of CD103$^-$CD11b$^+$ cDC2 cells was significantly increased in both obese and NASH mice compared with normal mice, while CD103$^+$CD11b$^-$ cDC1 cells were more abundant in NASH than those of obese livers.[16] In human NASH livers, an increased number of cDC1s (CD1c$^-$CD141$^+$XCR1$^+$) was also observed, which was highly associated with NASH pathologic scores.[16] Moreover, recent sequencing of physically interacting cells suggested that cDCs in NASH promoted the reprogramming of inflammatory T cells, which is linked to the worsening of liver pathology in NASH (Figure 5D).[16]

The development of therapy strategies

NAFLD pathologies can be largely reversed by physical activity, dietary interventions, and pharmacological treatment.[166] However, there is no approved pharmacotherapy for late-stage NAFLD with severe fibrosis or cirrhosis. The abovementioned NAFLD-associated cell types or subtypes offer valuable resources for drug target screening. For example, deletion of cDC1 in Xcr1^{DTA} mice abrogated liver pathology after methionine-deficient and choline-deficient diet feeding for 2 weeks.[16] Moreover, cell-type specific factors are excellent candidates for potential therapy targets, such as LPAR1 restrictedly to pathogenic collagen-producing HSCs[18] and OPN (coded by *Spp1*) secreted by TREM2$^+$ macrophages,[167] targeting which could attenuate NAFLD in mice.[18,167] Blockade of IL-15 and CXCR3, which were crucial for the maintenance of CD8$^+$ resident memory T cells, partially inhibited fibrosis resolution in mice.[17] On the other hand, scRNA-seq combined with perturbation studies has been employed to decipher the mechanism of antifibrotic drugs.[168–170] For example, elafibranor treatment markedly reduced the abundance of *Trem2*$^+$ macrophages,[113] and nizatidine, a histamine receptor H2 blocker, improved liver disease progression by targeting macrophages.[169] These studies highlight the power of single-cell studies in screening potential therapeutic targets.

LIVER CANCER

Liver cancer is a complex disease involving genomic alterations, viral infection, metabolic rewriting, and immune dysregulation.[171] Liver cancer has several pathological forms, with HCC being the most common form, followed by intrahepatic cholangiocarcinoma (ICC), mixed hepatocellular cholangiocarcinoma, and hepatoblastoma. The application of single-cell technologies and spatially resolved transcriptomics has greatly deepened our understanding of liver cancer in the aspects of tumor heterogeneity, tumor microenvironment (TME), cell communications, and tumor spatial architectures.[14,130,172–175]

The heterogeneity of malignant cells

When analyzed by scRNA-seq, malignant cells usually form patient-specific clusters on TSNE or UMAP, suggesting the high interpatient heterogeneity of malignant cells.[173,174] To measure tumor heterogeneity precisely, a sampling strategy for a given scRNA-seq data set was designed to measure the correlation of cell-type specific signatures of both malignant and nonmalignant cells before and after sampling.[130] As the number of patients increased, the signature of malignant cells changed, while those of nonmalignant cells were better retained once beyond 3 patients, demonstrating the higher interpatient variability of malignant cells than nonmalignant cells.[130] Furthermore, the heterogeneity of malignant cells was quantified by evaluating the overall variation in gene expression, which was defined as intratumor heterogeneity.[174] The intratumor heterogeneity score well predicts patient outcomes, with a high intratumor heterogeneity value associated with worse prognosis.[174]

In addition to interpatient heterogeneity, the intercellular heterogeneity of malignant cells has been unveiled through single-cell studies, such as malignant cell subpopulations defined by varied gene expression or copy number variant spectra,[176] circulating malignant cells at different vascular sites,[177] cancer stem cells subpopulations with various cell surface markers (CD24$^+$, EpCAM$^+$, CD133$^+$, and Triple$^+$),[178] and malignant cells with varied degree of proliferation.[48,179] These intercellular heterogeneities render remarkable effects on tumor progression.[48,178,179] For example, 4 malignant cell clusters representing G2/M, G1/S, G0. and an intermediate phase of cell cycle have been detected from scRNA-seq data set of 5 HCC cell

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lines through K-means analysis based on cell-cycle–related genes.[48] The increase in fraction of cells in G0 phase, which signified the decrease of proliferation capability, had a strong correlation with the decrease in metastatic potential.[48] Through scRNA-seq, investigators found that the proliferative subset (highly expressing G2/M genes) of Prom1$^+$ HCC cells in mouse models were significantly correlated to poor prognosis of HCC.[179]

Tumor immune microenvironment

Many efforts have been made to systematically depict the tumor immune microenvironment of liver cancer.[14,51,180,181] A recent scRNA-seq study profiling more than 1 million cells from 124 patients and 8 mice with liver cancer systematically delineated the immune landscape of liver tumor.[14] Moreover, there are another 3 global immune landscapes of HCC, covering about 77,000,[51] 41,700,[181] and 17,432,600[180] immune cells from 16, 7, and 13 patients, respectively. To uncover the molecular features of T cells, a recent study sequenced 5063 CD45$^+$CD3$^+$ T cells from the tumor, adjacent normal, and blood samples of 6 patients with HCC.[31] These scRNA-seq studies offer great details of tumor-infiltrating immune cells, including T cells, macrophages, neutrophiles, DCs, NK cells, and others,[14,51,180,181] providing comprehensive resources to understand immune landscape of tumor ecosystem and to interpret its roles in liver cancer.

Five tumor immune subtypes including immune activation, immune suppression mediated either by myeloid or stromal cells, immune exclusion, and immune residence phenotypes have been unraveled.[14] Notably, tumor-associated neutrophils enriched in the myeloid cell–mediated immune suppressive TME were associated with an unfavorable prognosis.[14] Meanwhile, *in vivo* neutrophil depletion in mouse models attenuated tumor progression, confirming the pro-tumor phenotypes of tumor-associated neutrophils.[14] Besides that, the comparison between tumor and adjacent normal samples also reveals tumor-associated immune cell subpopulations (Figure 6A). Exhausted CD8$^+$ T cells, *CTLA4*$^+$ CD8$^+$ T cells, *XCL1*$^+$ CD8$^+$ T cells, *PDCD1*$^+$ CD4$^+$ T cells, *FOXP3*$^+$ regulatory T cells, *CTLA4*^{high} regulatory T cells, *TREM2*$^+$ macrophages, *CD163*$^+$ macrophages, *CCL18*$^+$ M2 macrophages, *LAMP3*$^+$ DCs, *CCL4*$^+$ neutrophils, and *CD160*$^+$ NK cells have been reported to be preferentially enriched in tumor regions.[14,31,51,118,183] In contrast, cytotoxic CD8$^+$ T cells, *FGFBP2*$^+$*GZMB*$^+$ CD8$^+$ T cells, and cytotoxic NK cells have been found diminished in tumors (Figure 6A).[180] Rather than being static, the immune microenvironment dynamically changes during tumor progression (Figure 6A). Compared with primary tumors, relapsed HCCs carry a TME with increased CD8$^+$ T cells and DCs as well as reduced regulatory T cells and proliferative T cells.[21] It is worth noting that rather than exhibiting an exhausted state, CD8$^+$ T cells in relapsed HCCs were in a low cytotoxic status, offering a new mechanism of immune escape in relapsed tumors.[21] In addition, TME alters as tumor metastasizes.[184] *SPP1*$^+$ macrophages and *MRC1*$^+$ *CCL18*$^+$ macrophages have been found to be specifically increased in liver metastases of colorectal cancer.[184] Therapy intervention also changes the immune microenvironment.[173,184,185] ScRNA-seq reveals that radiotherapy can alter the TME by eliminating immunosuppressive hepatic macrophages and increasing hepatic T-cell infiltration.[185] In patients who respond to neoadjuvant chemotherapy, their TME transitioned to an antitumor state with increased cytotoxic T cells and decreased immunosuppressive cells, which was not observed in nonresponsive patients.[184]

In combination with deconvolution algorithms, the signature genes of these tumor-associated immune cell subtypes identified from single-cell studies allow us to infer immune cell proportions within the TME from bulk RNA-seq data sets.[21,31,180] Furthermore, when linked to clinical information, prognosis-associated immune cell types can be identified.[21,31,180] Based on this strategy, *XCL1*$^+$CD8$^+$ T cells have been found to be correlated with better survival and antitumor response,[180] while *CD161*$^+$CD8$^+$ T cells, *KLRB1*^{high} CD8$^+$ T cells, and *CCL18*$^+$ M2 macrophages have been linked to a poor prognosis.[21,180] Subtype-specific genes could be further examined to elucidate their roles in the TME. The expression of *LAYN*, a signature gene of exhausted CD8$^+$ T cells and tumor-specific Tregs, was significantly correlated with poor patient prognosis;[31] CCL5 had been shown to act as an important mediator for immune evasion of circulating tumor cells in the TME.[177]

Cell communications and spatial architectures

Cells within the tumor ecosystem do not operate in isolation but closely communicate with other cells.[186,187] Based on ligand–receptor pairs, the single-cell atlas facilitates the inference of cell–cell communication.[93] Within the interaction network between tumor cells and nonmalignant cells, cancer-associated fibroblasts and SAMs were identified as interaction hubs, representing 49.3% of all interactions.[130] Several recurring modules in the interaction network, including matrix remodeling, ERBB signaling, MET signaling, and WNT signaling, have been previously linked to tumor progression.[130] For tumor-immune interactions, PD-L1$^+$ or CTLA4$^+$ tumor cells showed recurrent interactions with CD80$^+$ DCs

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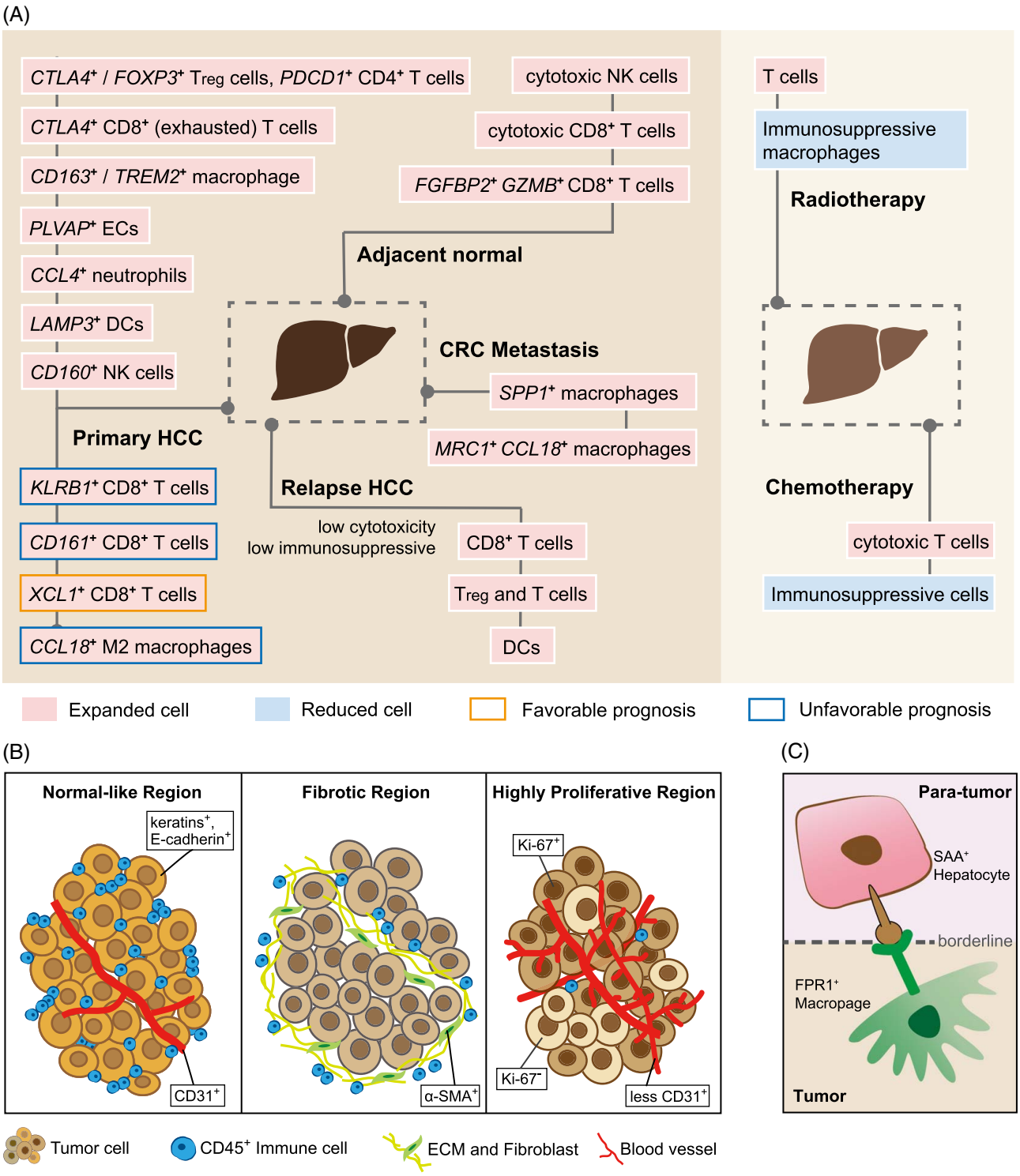

FIGURE 6 Tumor-associated cell populations and spatial architectures of liver cancer. (A) Tumor-associated cell populations in liver cancer. Left: immune or stromal cell populations enriched in adjacent normal tissues, primary HCCs, relapse HCCs, and liver metastases of colorectal cancer (CRC), respectively. Right: altered cell subpopulations on treatment. (B) Three major types of spatial architectures of HCC revealed by imaging mass cytometry.[175] Normal-like region is with strong staining for hepatic cell markers keratins and E-cadherin. Fibrotic region exhibits remarkable enrichment of ECM and α-SMA labelled fibroblasts. Highly proliferative region is characterized by the enrichment of Ki-67+ proliferative tumor cells and irregular blood vessels. (C) An example of cell-cell communication located at the tumor invasion front.[182] Abbreviations: DCs, dendritic cells; ECs, endothelial cells; ECM, extracellular matrix; NK cells, natural killer cells; Treg, regulatory T cell.

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and innate-like CD161$^+$ CD8$^+$ T cells, which might reduce the efficiency of antigen presentation and the activation of T cells.[21] A recent multiregional scRNA-seq analysis of 7 patients with liver cancer (4 HCCs and 3 ICCs) revealed that the ligand-receptor interaction between tumor cells and macrophages (LGALS9-SLC1A5 and SPP1-PTGER4) were consistently observed across multiple tumor regions and across multiple cohorts.[188] Notably, these communications were associated with tumor aggressiveness.[188] There are also extensive interactions between different immune cell types.[14,21,180,188] For example, PD-L1$^+$ tumor-associated neutrophils could directly interact with CD8$^+$ T cells, downregulating the expression of T-cell cytotoxic marker IFNγ and activation markers CD25 and CD69, thus mediating an immune-suppressive phenotype in liver cancer.[14] Furthermore, tumor lesions may communicate with other parts of the body through the immigration of immune cells.[51] Tumor-infiltrating *LAMP3*$^+$ DCs could migrate to hepatic lymph nodes, and subpopulations of lymphocytes and macrophages had been reported to migrate to ascites from tumors.[51] However, further investigation is needed to determine how these across-tissue communications affect liver cancer pathogenesis.

Importantly, the expeditious growth in spatial techniques promises to decode the spatial proximity of cells within the tumor ecosystem in a more direct and comprehensive way.[175,182,189] Applying imaging mass cytometry to 134 patients with HCC, Sheng et al [175] summarized 3 major types of intratumor regions carrying distinct topological features: (i) normal-like regions with highly expressed hepatocyte markers and intact blood vessels; (ii) fibrotic regions surrounded by immune cells; and (iii) highly proliferative regions with few immune cells and irregular blood vessels (Figure 6B). Furthermore, 16 topological units (also called cellular neighborhoods, CNs) were identified.[175] Some of these CNs were correlated with patient survival, with cancer cell–enriched CNs associated with unfavorable prognosis while CD8$^+$ T-cell–enriched CNs were associated with prolonged patient survival.[175] For the detailed spatial architecture, the leading edge of the tumor representing the invasive front has drawn much attention (Figure 6C).[15,130,181,182] Using bulk RNA-seq on laser capture microdissected regions of liver tumors, Massalha et al[130] found that SAMs and T cells were more abundant in the tumor border than in the tumor core. A progressive comparison from normal to leading-edge to tumor regions of 7 patients with liver cancer (5 HCCs, 1 ICC, and 1 mixed hepatocellular cholangiocarcinoma) was conducted based on 10X Visium, which revealed that complete tumor capsules consisting of fibroblasts and ECs might act as barriers preventing the infiltration of immune cells.[15] This study also found that tertiary lymphoid structures mainly existed in leading-edge regions and that the distinct composition of tertiary lymphoid structures might be shaped by their distance to tumor regions.[15] Moreover, a single-cell study involving an analysis of 39 matched HCC leading-edge and nontumor specimens by mass cytometry observed the enrichment of CD4/CD8 double-positive T cells in leading-edge regions, which is significantly associated with good prognosis.[181] Another study on ICC found increased expression of SAA in hepatocytes close to the invasive front of tumors, which might recruit *FPR1*$^+$ macrophages to aid tumor invasion (Figure 6C).[182]

CHALLENGES AND PERSPECTIVES

In this review, we described the applications of single-cell and spatial techniques to liver biology and summarized the progress in this field at unprecedented resolution, covering topics about formation and maintenance of the liver, liver regeneration, pathogenesis of NAFLD, and liver cancer. Relying on single-cell and spatial techniques, investigators have uncovered extraordinary diversity in cell populations, state transitions, functions, cell-to-cell communications and spatial architectures within the liver, providing novel insights into liver zonation,[125,127] cell plasticity,[10] microenvironment reprogramming,[5,8] disease-associated cell populations,[5,11,16] and tumor topological structures.[15]

Several outstanding challenges still need to be addressed. (i) A specialized single-cell analysis protocol considering the characteristics of liver tissue remains to be established. PCs and NPCs exhibit very different features after sequencing due to their distinct morphologies and functions.[29,33,34] Compared with NPCs, large cell size (about 20–30 μm in diameters) and active metabolism of hepatocytes lead to increased library size and mitochondrial counts as well as far more ambient mRNA.[20,29] When rendered quality control, the same filtering criteria would cause cell loss mainly in hepatocytes.[29] In our opinion, a 2-round data preprocessing would help resolve the question. Investigators could first filter data with loose cutoffs, simply perform clustering, and identify PCs and NPCs according to well-known markers, and then, different criteria could be applied to PCs and NPCs to improve the downstream analysis. Moreover, it is necessary to filter out ambient RNAs. Tools such as SoupX[190] and DecontX[191] would help remove these contaminants. (ii) How to determine cell identity, especially rare or intermediate state, remains challenging. Standardized cell ontology of hepatic cells including common, rare, and disease-related cell populations is urgently needed. It is very necessary to build a grand liver single-cell atlas that well integrates the abundant

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published resources.[20,23,192] This would greatly empower the establishment of the standardized cell ontology, the discover of undefined cell state/identity, and the annotation of newly generated data sets. (iii) The highly organized spatial architectures of the liver at single-cell resolution and their roles in liver diseases remain to be elucidated. Current spatial techniques have limited capacity to juggle resolution and coverage.[55] Computational strategies integrating spatial omics data with single-cell data[108] and mathematic algorithms modeling spatial changes[193,194] are highly needed. They would help interrogate gene expression changes across lobular zones, decode cell communications in-deep within niches of interest, and uncover new spatial features. In addition, there are emerging new spatial technologies offering single-cell or sub-cell resolution.[64,195] These will boost the discovery of new insights in the field. (iv) Clinical applications, such as translating research findings into novel therapeutic approaches, are still in their infancy. For example, only countable features from the aforementioned studies are recognized as potential therapeutic targets (PGLS and MLXIPL) for HCC.[196–198] Experimental validation and further exploration should be emphasized.

Experimental technologies and computational strategies offering multiomic or multimodal solutions merit more attention.[199,200] Multiomic techniques such as CITE-seq and NanoString CosMx Spatial Molecular Imager have already unveiled new insights into chronic liver diseases.[5,45,46,195] Joint profiling of scRNA-seq with scATAC-seq or DNA methylation allows to delineate the principles of gene regulation during cell state/identity transition.[201,202] Despite these, new multimodal integration strategies are still needed. For example, there is still limited strategy to integrate scDNA-seq, an insightful technology to study tumor evolution,[203,204] and scRNA-seq. In addition, bulk-sample based studies have presented abundant phenotypes from big cohorts.[205,206] The integration of bulk data, scRNA-seq, and spatially resolved transcriptomics can link phenotype information, cell status, and spatial locations together. However, there is currently no generally accepted strategies to conduct such integrative analysis yet.

We believe that new techniques and computational approaches will further lead us to the full picture of the cell landscape, the molecular dynamics, and the spatial architectures of the liver, enabling the identification of novel therapeutic targets across the spectrum of liver diseases.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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